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13. ABSTRACT *(Maximum 200 Words)*

The research objective outlined in the original proposal was to determine the role of the integrin-linked kinase (ILK) in the induction and progression of metastatic mammary tumors. This work was initially based on the observation that overexpression of ILK in cultured epithelial cells results in changes characteristic of oncogenic transformation (Hannigan *et al.*, 1996; Novak *et al.*, 1998; Radeva *et al.*, 1997; Wu *et al.*, 1998). In order to evaluate the oncogenic potential of ILK in the mammary gland, we derived transgenic mice that express ILK under the transcriptional control of the MMTV promoter. The appearance of focal mammary tumors in these MMTV/ILK mice confirms that mammary-specific expression of ILK can facilitate malignant transformation *in vivo*. A second major objective was to determine the role of ILK in tumor progression by ablating its function. For this purpose, we established transgenic mice expressing a kinase-dead allele of ILK in the mammary epithelium. We are currently breeding these mice with our MMTV/erbB-2 strains to assess the importance of ILK in erbB-2-mediated mammary tumorigenesis and metastasis. This last experiment is particularly relevant to the understanding and treatment of human breast cancer, given the known importance of erbB-2 in the progression of this disease.

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D. White 6/6/00

PI - Signature Date

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INTRODUCTION

The research objective outlined in the original proposal was to determine the role of the integrin-linked kinase (ILK) in the induction and progression of metastatic mammary tumors. This work was initially based on the observation that overexpression of ILK in cultured epithelial cells results in changes characteristic of oncogenic transformation, including anchorage-independent growth, suppression of cell death in suspension, invasion of extracellular matrices, and tumorigenicity in nude mice (Hannigan *et al.*, 1996; Novak *et al.*, 1998; Radeva *et al.*, 1997; Wu *et al.*, 1998). In order to evaluate the oncogenic potential of ILK in the mammary gland, we have derived transgenic mice that express ILK under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer. The appearance of focal mammary tumors in these MMTV/ILK mice demonstrates that the mammary-specific expression of ILK can facilitate malignant transformation. A second major objective presented in the proposal was to determine the role of ILK in tumor progression by ablating its function. For this purpose, we established transgenic mice expressing a kinase-dead allele of ILK in the mammary epithelium. We are currently breeding these mice with our MMTV/erbB-2 strains to assess the importance of ILK in erbB-2-mediated mammary tumorigenesis and metastasis. This last experiment is particularly relevant to the understanding and treatment of human breast cancer, given the known importance of erbB-2 in the progression of this disease.

RESEARCH ACCOMPLISHMENTS

1) Establishment and characterization of a transgenic mouse expressing ILK in the mammary epithelium

In order to examine the impact of ILK overexpression *in vivo*, we generated 3 independent founder lines of mice expressing the full-length ILK cDNA in the mammary epithelium, under the transcriptional control of the MMTV long terminal repeat (LTR) (figs 1A and 1B, appendix 1). Although ILK is already expressed endogenously in the murine mammary gland, the MMTV-LTR promoter/enhancer would ensure a constitutive level of expression beyond that normally found in the glandular epithelium. Tissue-specific transgene expression was confirmed by RNase protection analysis, revealing expression in salivary and mammary glands of female mice, as well as seminal vesicles and epididymus of males (fig. 1C), consistent with that reported in other MMTV-based transgenic models (Dankort and Muller, 1996; Muller *et al.*, 1990; Muller *et al.*, 1988). An RNase protection probe to PGK was used as an intermal control for RNA levels and integrity.

The mammary glands of virgin mice expressing the MMTV/ILK transgene appeared normal at 8 to 12 weeks of age, according to whole-mount and histological examination (not shown). From the ages of 12 to 24 months, however, focal mammary tumors appeared in female mice from all 3 founder lines, at an incidence of 39% (figs 2 and 3). Examination of tumor and lung tissue sections revealed invasive mammary adenocarcinomas, which appear to metastasize to the lung in 50% of the cases (fig. 3). These results demonstrate that elevated expression of ILK can predispose cells of the mammary epithelium to malignant transformation. However, because the mammary tumors were focal in nature and arose after a long latency period,

expression of ILK alone is likely not sufficient to induce mammary tumors. Rather, these observations suggest that tumor progression in these MMTV/ILK mice requires additional genetic events.

In a routine analysis, we measured the levels of ILK protein in both tumor tissue and normal adjacent mammary gland taken from the MMTV/ILK mice. The results of these analyses revealed that the tumor tissue from the MMTV/ILK mice expressed elevated levels of ILK, by comparison to the adjacent normal epithelium (fig. 4, top panel). Because elevated levels of ILK have been associated with increased levels of cyclin D1 in culture (Radeva *et al.*, 1997), we then performed immunoblot analyses with cyclin D1 specific antibodies, confirming that the tumor samples indeed expressed elevated levels of cyclin D1 protein (fig. 4, bottom panel). Given the elevated expression of both ILK and cyclin D1 in these tumors, it is conceivable that co-expression of these proteins is required for efficient tumor induction in these MMTV/ILK mice.

In addition to cyclin D1, another important signaling molecule that is thought to play a role in ILK-induced transformation is the serine kinase PKB/Akt. Previous biochemical analyses in epithelial cell culture have revealed that Akt is phosphorylated on serine 473 in response to ILK overexpression (Delcommenne *et al.*, 1998). Using immunoblot analysis, with antibodies specific to phosphorylated serine 473 of Akt, we showed that Akt is also phosphorylated at elevated levels in the MMTV/ILK mice, as compared to FVB controls (fig. 5). These observations confirm that Akt is indeed a downstream target of ILK *in vivo*, recapitulating the results seen in cell culture.

2) Expression of a kinase-dead allele of ILK in the mammary epithelium

A second major goal during the last funding period was to generate transgenic mice expressing a kinase-dead allele of ILK in the mammary epithelium. In collaboration with Dr. Shoukat Dedhar, British Columbia Cancer Agency, Vancouver, we placed a kinase-dead (KD) version of ILK (Novak *et al.*, 1998) in the MMTV expression cassette. The resulting MMTV/ILK-KD construct was then injected into the pronuclei of fertilized eggs, and RNase protection analysis was subsequently used to confirm expression of the transgene in the resulting ILK-KD line #1414 (fig. 6). These mice are currently being analysed for the presence of mammary gland abnormalities, while further injections are planned in order to derive more ILK-KD founder lines. In the meantime, the MMTV/ILK-KD mice have been crossed with those expressing activated erbB-2/neu in the mammary gland, in order to determine the requirement for ILK activity in growth factor-induced oncogenesis, as discussed below.

3) The use of MMTV/erbB-2 mouse models to elucidate the role of ILK in mammary tumorigenesis.

In order to elucidate the role of ILK in mammary tumorigenesis, we have also taken advantage of existing animal models expressing various oncogenes in the mammary epithelium. In this regard, we first looked at the role of endogenous ILK in mammary tumorigenesis by performing immunoblot analyses on mammary tumors taken from mice expressing various alleles of activated erbB-2/neu (Siegel *et al.*, 1999), using antibodies specific for ILK. The

results of these experiments revealed that tumors induced by activated erbB-2 expressed elevated levels of ILK, particularly in contrast to tumors resulting from alleles of erbB-2 lacking tyrosine residues which are necessary for the activation of downstream signaling pathways (Dankort *et al.*, 1997) (fig. 7). Although all of the erbB-2 alleles can transform the mammary epithelium, the resulting tumors differ in terms of kinetics and metastatic potential. A direct role for ILK in growth factor signaling has previously been suggested by experiments in cell culture (Tu *et al.*, 1998; 1999), and a specific correlation between ILK and erbB-2 expression has been observed in mouse epidermis (Xie *et al.*, 1998). Given, therefore, the possible role of ILK in erbB-2-induced mammary tumor progression, we are currently breeding the MMTV/ILK with the MMTV/erbB-2 mice to determine whether elevated expression of ILK can accelerate mammary tumor progression and promote metastasis in these strains. In addition, we are interbreeding the MMTV/erbB-2 mice with those expressing the MMTV/ILK-KD construct, to determine whether ILK activity is required for growth factor-mediated tumor induction and progression. The results of these crosses are not yet available, but should provide important insight into the role of ILK in erbB-2 induced tumorigenesis.

4) Co-expression of ILK does not compensate for a lack of PI-3' kinase signaling in PyV mT-induced tumorigenesis

The original proposal also described an experiment to determine whether elevated expression of ILK could complement a mutant PyV mT oncogene that is decoupled from the PI-3' kinase signaling molecule (Webster *et al.*, 1998), given that ILK is thought to be a direct downstream target of PI-3' kinase (Delcommenne *et al.*, 1998). Complementation of the mT mutant by ILK would be revealed by an increase in mutant mT-induced tumor kinetics and metastasis, to levels seen in mice expressing the wild-type PyV mT antigen, which signals through PI-3' kinase (Webster *et al.*, 1998). This experiment was performed by breeding the MMTV/ILK mice with those expressing the mutant mT antigen, also under control of the MMTV promoter. The experiment was designed ultimately to confirm the importance of ILK in the PI-3' kinase/Akt pathway *in vivo*. The results of this experiment, however, revealed that elevated expression of ILK was not sufficient to complement the defect observed in the PyV mT strains, in terms of tumor kinetics and metastasis (fig. 8). These results would suggest that perhaps there is a requirement for other PI-3' kinase targets, such as PDK1 and the Rac/Rho small GTPases (Currie *et al.*, 1999; Rodriguez-Viciana *et al.*, 1997), for the induction of metastatic tumors resembling those induced by the wild-type mT antigen. Perhaps experiments with more suitable mammary tumor models, such as those described in the previous section, may be more appropriate in addressing the role of ILK in mammary tumor progression.

TRAINING ACCOMPLISHMENTS

Over the past year, I have acquired skills pertaining to the construction and analysis of transgenic mice. These include the underlying molecular biological manipulations, as well as biochemical analysis and immunohistochemistry. In addition to these basic laboratory skills, I have gained a great deal of knowledge regarding the histology and histopathology of normal and cancerous mammary gland tissue. I now have a basic understanding of the progression of

metastatic disease, including the anatomical distribution of breast cancer metastases, as well as the underlying molecular mechanisms of invasion and metastasis. This knowledge has come from working not only with my supervisor, Dr. William J. Muller, but also with our pathologist, Dr. Robert Cardiff of the University of California, Davis. Dr. Cardiff is only one of several collaborators which we can access as a resource in Dr. Muller's lab.

KEY RESEARCH ACCOMPLISHMENTS

- **Generation and characterization of mice expressing ILK in the mammary epithelium, under control of the MMTV promoter, and demonstration of tumorigenesis in those mice**
- **Generation and characterization of mice expressing a kinase-dead allele of ILK, also in the mammary epithelium, and under control of the MMTV promoter**
- **Interbreeding of MMTV/ILK and MMTV/ILK-KD mice with MMTV/erbB-2 mice, to assess role of ILK in mammary tumorigenesis**

REPORTABLE OUTCOMES

- **Abstract--Oncogene Meeting, Salk Institute, San Diego, California, June 22-25, 2000 (see abstract, appendix 2)**

CONCLUSIONS

Using a transgenic approach, we were able to test directly the impact of ILK overexpression and ablation *in vivo*, taking advantage of the mouse mammary gland as a well characterized tissue, easily amenable to manipulation and analysis, and widely used as a model system for the evaluation of developmental and oncogenic processes (Dankort and Muller, 1996; Hennighausen, 2000).

Several important results have been reported in the first year of funding for this project. Firstly, we have demonstrated that mammary epithelial expression of ILK is capable of inducing metastatic mammary tumors. However, because these tumors arise in a stochastic fashion and are focal in origin, mammary-specific expression of ILK does not seem to be sufficient for mammary tumor progression. Given the role of Akt as an important member of a cell survival pathway (Alessi *et al.*, 1996; Brunet *et al.*, 1999; Delcommenne *et al.*, 1998; Franke *et al.*, 1997; Webster *et al.*, 1998), it is conceivable that activation of Akt by ILK may be involved in the observed mammary tumor phenotype in the MMTV/ILK strains, perhaps by providing an anti-apoptotic background for the growth and proliferation of cells which have undergone a second genetic event. Alternatively, ILK may be influencing tumor progression through the upregulation of cyclin D1 and promotion of the cell cycle, since elevated ILK levels are accompanied by elevated cyclin D1 levels in the MMTV/ILK-induced tumors. Further studies designed to assess the relative contribution of these signaling pathways may provide important insight into the molecular basis for ILK induced tumorigenesis. Regardless, a possible requirement for additional genetic events would mean that the MMTV/ILK mice may provide a useful model for the study of multi-step oncogenesis, particularly through interbreeding experiments with other

MMTV/oncogene models.

In addition to overexpression in the mammary gland, we have started to address the role of ILK in mammary tumorigenesis through the construction of a mouse expressing a kinase-dead allele of ILK in the mammary epithelium. These mice have been bred with those expressing an activated allele of erbB-2 in order to assess whether ILK function is required for erbB-2 induced mammary tumorigenesis. The rationale behind this experiment is reinforced by our observation that erbB-2 induced mammary tumors express elevated levels of ILK. The results of such experiments should have important implications for both the diagnosis and treatment of human breast cancer, particularly given the well established role of erbB-2 in this disease.

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Appendix 1

Figures 1-8

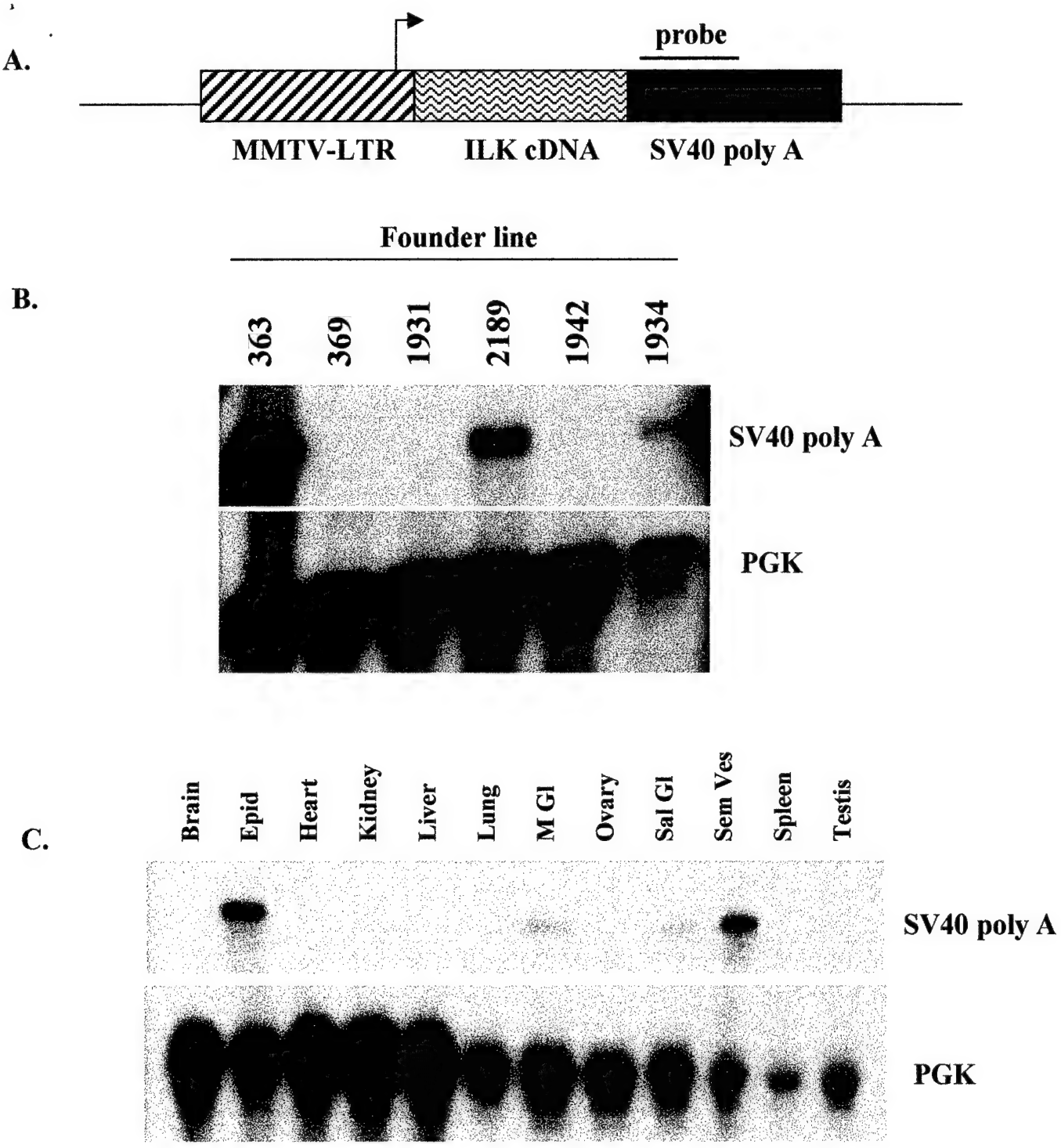

Figure 1. Tissue-specific expression of ILK in the mammary gland of transgenic mice. (A) Structure of the ILK transgene. (B) Expression of the ILK transgene in the mammary epithelium of 3 founder lines, as assessed by RNase protection. (C) Tissue distribution of transgene expression in both male and female mice. RNA antisense probes produce protected fragments of the poly adenylation region from SV40 (SV40 poly A), as well as that of a PGK internal control. M. Gl: mammary gland; Sal. Gl: salivary gland; Sem. Ves: seminal vesicle.

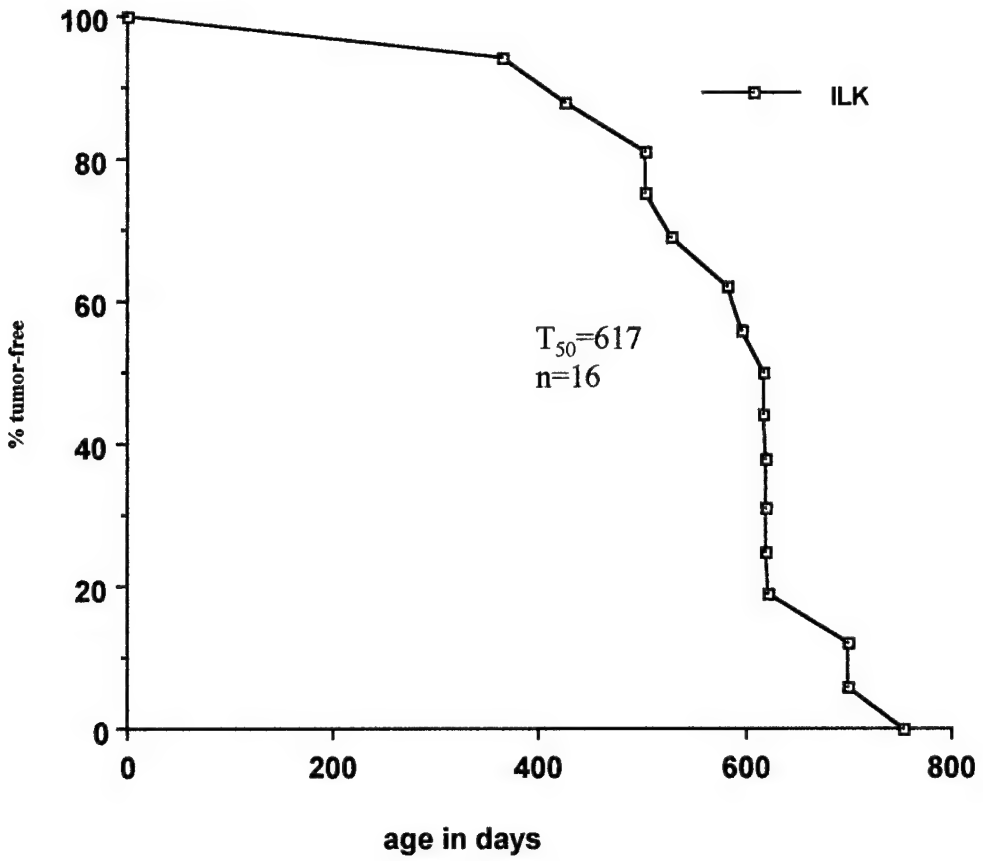

Figure 2. Kinetics of tumor formation in MMTV/ILK mice, line 363. Median age of onset (T_{50}) is 617 days, as determined from 16 tumor-bearing mice (n=16). Tumor incidence was calculated as 39% (16/41).

A.

B.

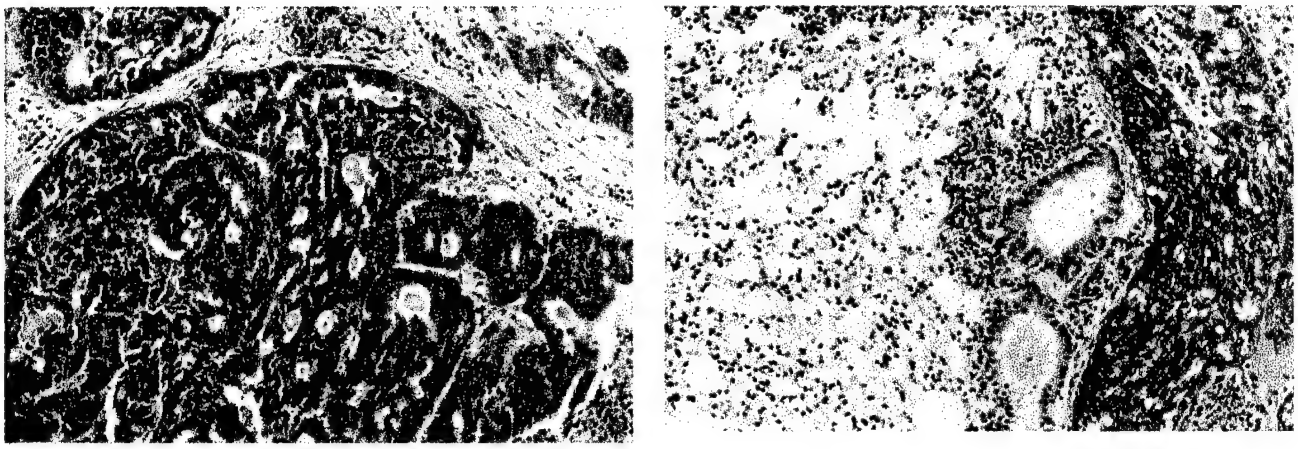

Figure 3. Hematoxylin and eosin stained tissue sections of (A) mammary adenocarcinoma and (B) lung taken from female mouse expressing the MMTV/ILK transgene (200x).

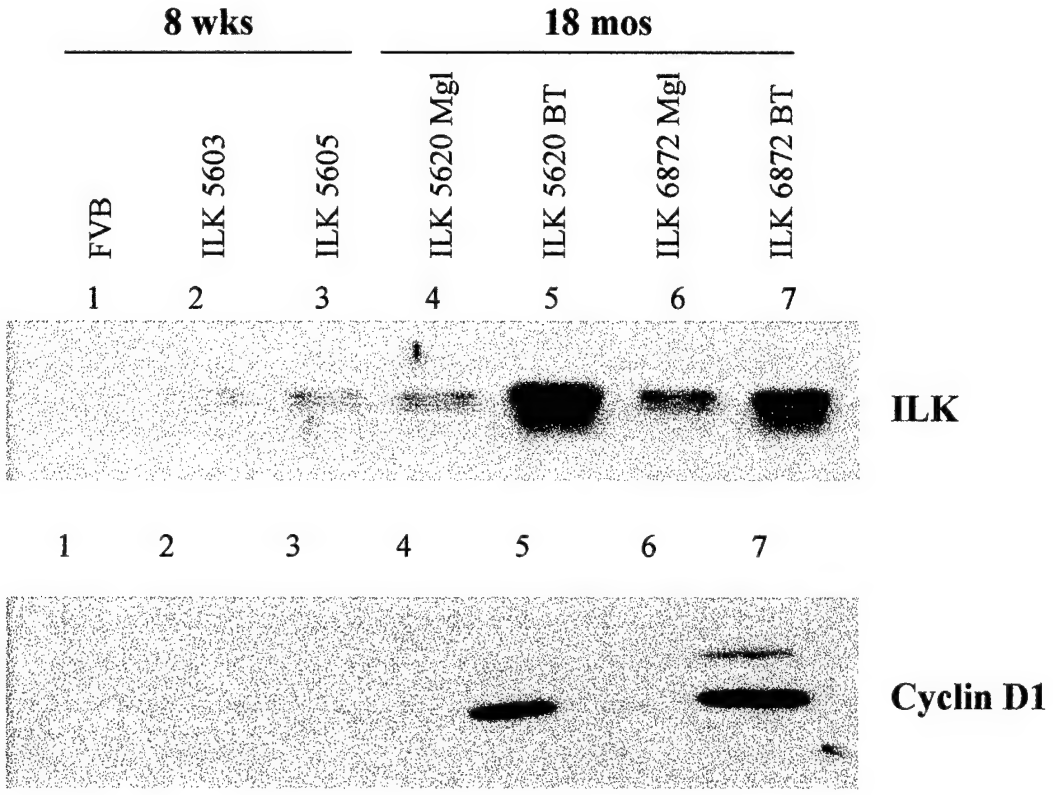

Figure 4. Upregulation of ILK and cyclin D1 in mammary tumours induced by MMTV-ILK transgene expression. Representative immunoblot showing protein levels in 5ug of normal virgin FVB (lane 1) and transgenic glands, aged 10 weeks (lanes 2,3), and in tumour (lanes 5,7) and adjacent mammary gland (lanes 4,6) tissue taken from 2 mice, aged 18 months. All transgenic mice are of the line #363, and are indicated by the label ILK, plus the ear-tag number.

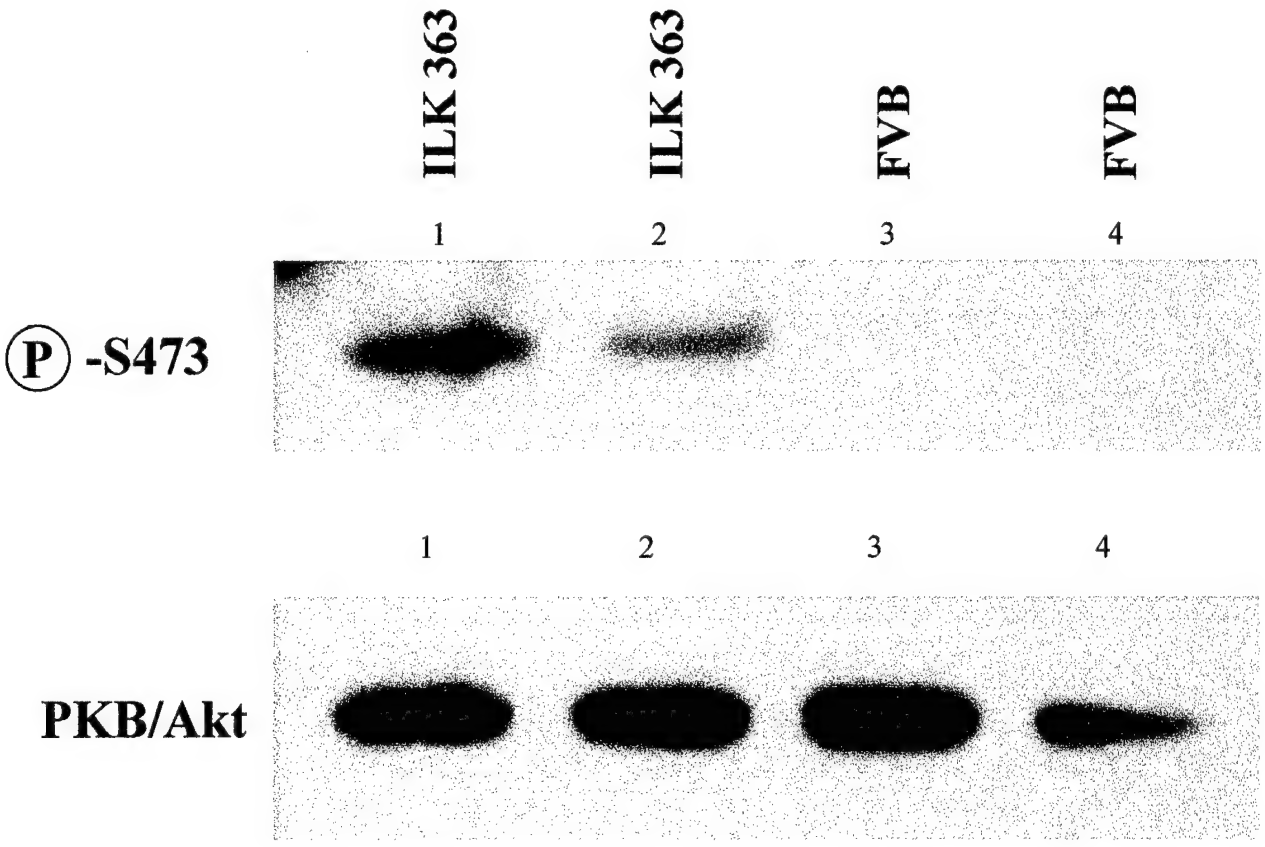

Figure 5. Comparison of phospho-Akt (S473) levels between mammary glands of 2 female ILK transgenic mice (line #363) (lanes 1,2) and 2 FVB control mice (lanes 3,4), 8 weeks of age. The lower panel shows total Akt levels in the same samples.

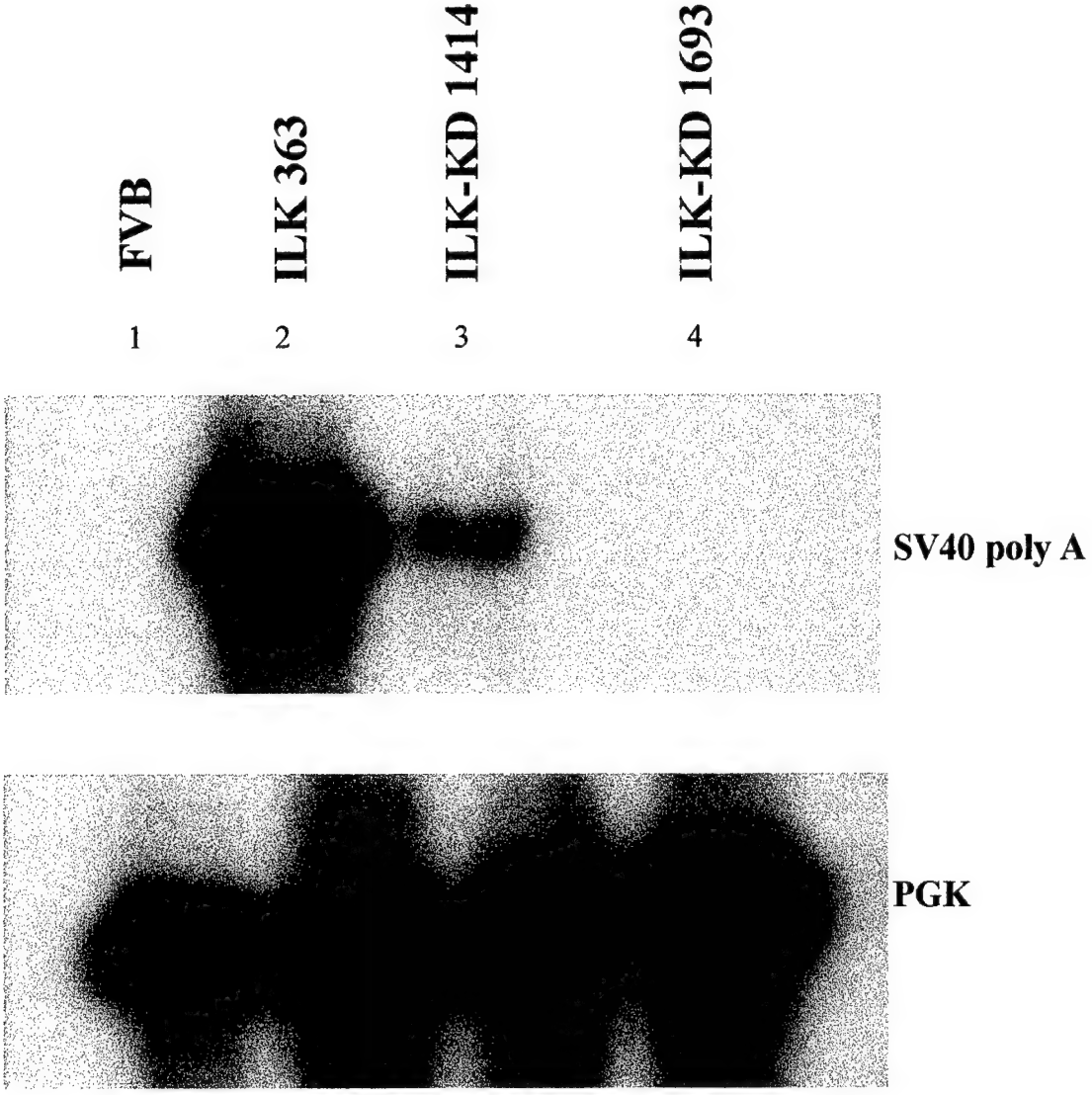

Figure 6. Expression of a kinase-dead (KD) allele of ILK in the mammary epithelium. Expression of an MMTV-ILK-KD transgene in the mammary epithelium of line #1414 was confirmed by RNase protection (lane 3). An FVB control and a non-expressing founder (lanes 1,4), as well as a mouse expressing the wild-type ILK transgene(lane 2), are shown as negative and positive controls for transgene expression, respectively. The KD allele of ILK was provided by Dr. Shoukat Dedhar of the University of British Columbia. This allele contains a glutamic acid to lysine substitution at position 359, in the conserved kinase domain of ILK (Novak et al., 1998). The mutant cDNA was cloned into an expression vector, downstream of the MMTV-LTR, and injected into the pronuclei of fertilized egges. Protection of phosphoglycerate kinase (PGK) transcripts are shown as internal controls for RNA integrity.

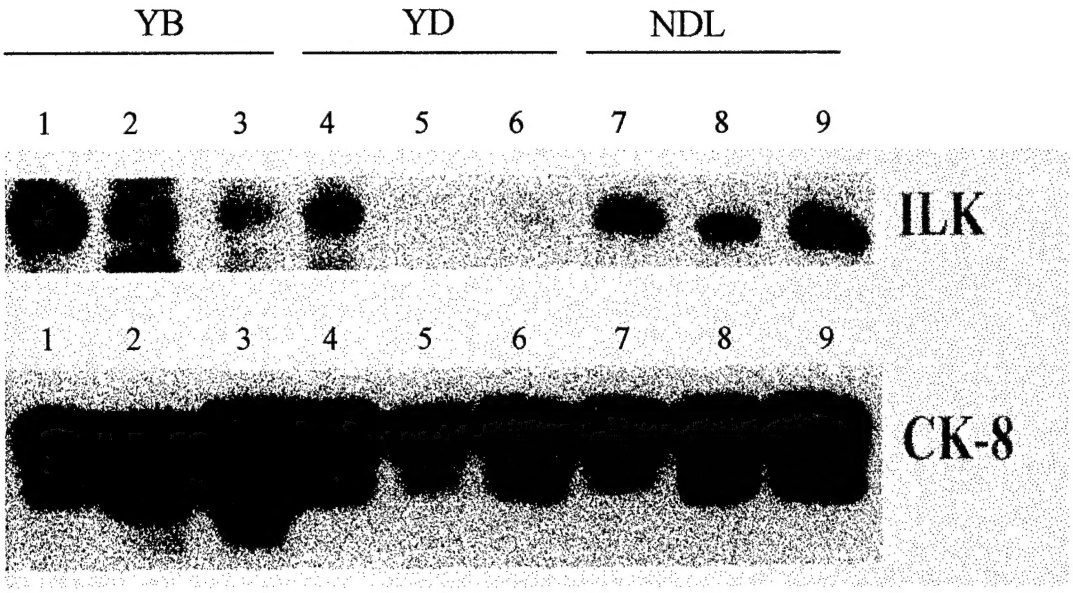

Figure 7. ILK levels are elevated in tumors expressing an activated erbB2/neu allele (NDL) containing all five tyrosine residues known to be phosphorylated in response to erbB2/neu kinase activity (lanes 7,8,9). By comparison, tumors expressing an allele deficient in all tyrosine residues except for site D (YD) have relatively lower levels of ILK (lanes 4,5,6). These YD-induced tumors have a longer latency than those induced by the activated NDL allele, and are less metastatic. An allele containing only tyrosine residue B (YB) induces tumors which, by comparison, are more metastatic to those induced by the YD allele, and appear earlier. ILK levels in the YB-induced tumors are shown in lanes 1,2 and 3. A cytokeratin-8 (CK-8) blot (lower panel) provides a control for protein loading and epithelial cell content.

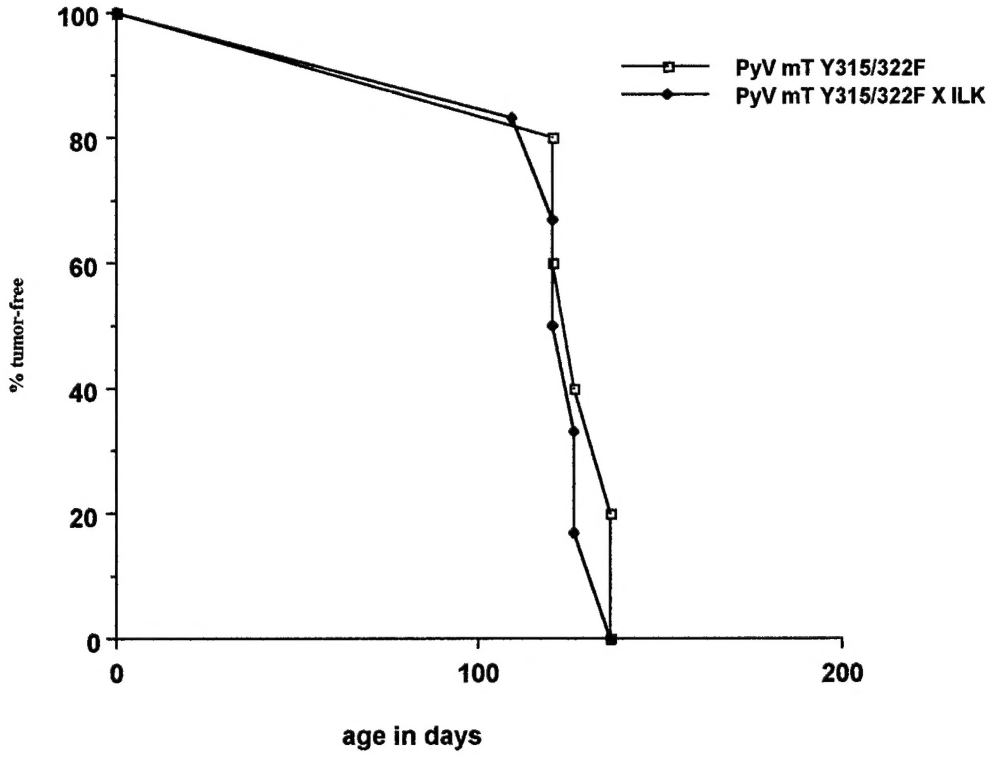

Figure 8. Kinetics of tumor formation in MMTV/PyV mT Y315/322F mice (n=5), versus MMTV/PyV mT Y315/322F x MMTV/ILK bitransgenic mice (n=6).

Appendix 2

Meeting Abstract

Tumorigenesis in Transgenic Mice Expressing the Integrin-Linked Kinase (ILK) in the Mammary Epithelium

Donald E. White[1], Shoukat Dedhar[3], Robert D. Cardiff[4] and William J. Muller[1,2]

Departments of Medical Sciences[1] and Pathology[2], McMaster University, Hamilton, Ontario, Canada, British Columbia Cancer Agency and Jack Bell Research Centre[3], Vancouver, British Columbia, Canada, and the Department of Pathology[4], School of Medicine, University of California, Davis, California, USA

The integrin-linked kinase (ILK) is a 59K serine-threonine kinase, identified by virtue of its association with the cytoplasmic domains of β1- and β3-integrins. Transformation of cultured epithelial cells by overexpression of ILK suggested that ILK might contribute to tumorigenesis, invasiveness and metastasis *in vivo*. In order to test this hypothesis in a physiological context we generated mice expressing the full-length ILK cDNA in the mammary epithelium, under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat. Focal mammary tumors appeared in 36% of female animals between the ages of 18 and 24 months, and pulmonary metastases were observed in 50% of these mice. In addition, increased phosphorylation of PKB/Akt on serine 473 was confirmed by immunoblot analysis of whole mammary gland, recapitulating the PKB/Akt-specific phosphorylation observed following ILK overexpression in culture. These experiments possibly provide the first direct demonstration of ILK's potential to induce tumorigenesis when overexpressed *in vivo*.